Dysfunctional Activation and Apoptotic Fragility in Hem1-Deficient B Cells

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Abstract

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Loss-of-function mutations in *NCKAP1L* and its associated protein, HEM1, cause severe primary immunodeficiency (PID) in children associated with mature B lymphopenia and impaired humoral immune response to foreign antigens, but also with B cell hyperactivation and production of autoantibodies. However, the mechanism by which loss of HEM1 causes this phenotype in B cells is poorly understood. In this study, we examined increased activation and metabolic activity in Hem1-deficient murine B cells from a transgenic conditional knockout mouse model. By fixing the B cell receptor repertoire using MD4 (IgHEL), we were able to confirm higher activation in Hem1-deficient B cells independent of BCR specificity. We demonstrate that this hyperactivated phenotype is directly associated with vulnerability to apoptosis, where risk of cell death rises with increasing strength of BCR activation. We observed increased mitochondrial mass and ROS in Hem1-deficient cells, which may both increase signal

strength and promote cytotoxicity leading to apoptosis. Our results are supported by RNASeq validated by RT-qPCR, which showed upregulation of genes associated with growth, metabolism, proliferation, and DNA synthesis following disruption of Hem1.

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LIST OF ABBREVIATIONS

ARP2/3- Actin related protein 2/3

BCR – B cell receptor

F-actin – Filamentous actin

FO – follicular

Hem1 - Hematopoietic protein-1

HEL – hen egg lysozyme

IgD – Immunoglobulin D

IgM – Immunoglobulin M

KEGG - Kyoto Encyclopedia of Genes and Genomes

MFI – median fluorescence intensity

MZ – marginal zone

Nckap1l – Nck-associated protein1-like

PID – primary immunodeficiency disease

PCA - principal component analyses

ROS – reactive oxygen species

SOX - superoxide

TLR – toll-like receptor

VCA - combined Verprolin-homology domains (V), Cofilin domain (C), and acidic domain (A)

WASP – Wiskott-Aldrich syndrome protein

WAVE – WASP-family verprolin homologous protein

WT – wild-type

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DEDICATION

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I. Introduction

B cells are heavily featured lymphocytes in the adaptive immune response, defined by their ability to recognize antigens and produce antibodies against them. Naïve B cells express hundreds of thousands of identical copies of their B cell receptor (BCR), which recognize specific antigens represented as sequences of amino acids. At this stage, the BCR is expressed only as IgM, or the relatively less sensitive isoform, IgD (1). Upon encountering cognate antigen, stimulation of the BCR and its coreceptors causes a cascade of changes in the B cell's physiology and behavior. A newly activated B cell will grow and proliferate rapidly and may undergo BCR tuning and/or antibody class switching in order to bind its cognate antigen more effectively (1). After activation, class-switched BCRs may be secreted into serum as antibody, which may interact with antigens independently of the B cell itself (2).

However, for the adaptive immune response to serve its intended functions, it requires immune cells to be activated only by foreign threats, and never by the host's own antigens. Under normal circumstances, this balance is achieved through multiple mechanisms of tolerance. B cell tolerance is maintained during central development by clonal deletion, receptor editing, and targeted deletion of cells expressing self-reactive BCRs (2). Still, a small number of autoreactive mature B cells are always present in the periphery of healthy individuals despite expressing BCRs that bind host tissues. A number of processes are utilized to help maintain B cell tolerance in the periphery, including functional anergy by way of BCR downregulation and increased susceptibility of self-reactive B cells to apoptosis (2). Typically, although these cells may persist in the body, their ability to detect and signal detection of cognate antigen is greatly diminished. Yet despite the numerous layers of protection in place against the proliferation of self-reactive B cells, it is still possible for tolerance to be broken, resulting in systemic autoimmunity and autoimmune disease. Mysteriously, this phenomenon may occur even alongside severe immunodeficiency, as seen in patients with defects in WAVE regulatory complex (WRC) (3).

In recent years, nine individuals from seven independent kindreds were identified with severe primary immunodeficiency (PID) associated with loss-of-function mutations in *NCKAP1L* and its associated protein, HEM1, a hematopoietic cell-specific subunit of the WRC (3-6). In B cells, HEM1 and other WRC proteins exist in an autoinhibited resting state until induced into an

active conformation by immunoreceptor engagement with antigen or ligand (7, 8). Downstream of antigen/ligand binding, multiple phosphorylation events occur in the cytosol, leading to cleavage of the SRA1-VCA dimer and recruitment of the WRC to the plasma membrane, where F-actin is synthesized (Figure 1). As a foundational member of the WRC, HEM1 plays a vital role in early activation of the WRC in hematopoietic immune cells, and its deletion has devastating effects on health. With *HEM1* disrupted, lymphocytes and other immune cells are unable to produce normal F-actin, and experience difficulties in maturation, chemotaxis, adhesion, and phagocytosis (3-8).

Mice with loss-of-function mutations in *Nckap1l* experience profound lymphopenia, with particularly devastating effects on numbers of mature B cells (7-11). When *Hem1* was disrupted in mice either constitutively or in a B cell specific manner, B cells exhibited stunted development, leading to a near-total absence of marginal zone B cells in spleen, and reduced recirculating follicular B cells in bone marrow and periphery. Hem1-deficient B cells migrated poorly when exposed to a chemokine gradient, and produced dysfunctional antibody responses. With Hem1 disrupted, antibody responses were diminished against T-independent antigens, but were simultaneously enriched against T-dependent antigens (10).

Because few mature MZ and FO B cells are available to detect and mount a response against threats, and those that remain may have an impaired ability to do so, human patients with HEM1 dysfunction suffer from frequent infections and high mortality. However, many of these patients also report systemic autoimmune disease (3-6). Paradoxically, the same cells that are unable to effectively respond to exogenous threats seem to be hyper-responsive to host tissues.

In this study, we explored potential causes of the hyperactivated, B lymphopenic phenotype associated with specific loss of Hem1 in B cells. We observed B cell hyperactivation which occurred independently of BCR specificity, and resulted in increased sensitivity of Hem1-deficient B cells to apoptosis and cell death. In addition, we demonstrate altered mitochondrial metabolic activity following exposure of Hem1-deficient B cells to stimulation with TD and TI antigens. These results are supported by RNASeq and RT-qPCR analyses indicating increased expression of genes associated with cell growth, proliferation, and metabolism.

II. Results

Hem1 deficiency results in increased B cell activation independent of BCR specificity.

We had previously shown that disruption of Hem1 in B cells resulted in increased expression of activation markers CD25 and CD69, both at baseline and following α-IgM stimulation. We hypothesized that the increased B cell activation following disruption of Hem1 could be due to: 1) weaker B cell immune synapse formation, resulting in B cell selection during B cell development where stronger signaling self-reactive BCRs are selected for during positive selection, and/or 2) increased BCR interactions caused by decreased cortical actin formation (Figure 8). To examine these differential hypotheses, we bred *Hem1floxed* mice to *Mb1Cre* mice, which express Cre recombinase throughout B cell development from the pro-B cell stage (11). We then bred *Hem1floxedMb1Cre* mice to *MD4* transgenic mice, which express the monoclonal Immunoglobulin heavy and light chain against hen egg lysozyme (HEL), a non-self antigen (12). Because all B cells express identical IgM and IgD with a single antigen specificity, BCR selection is negated (Model 1) thus allowing us to examine the effects of Hem1 deletion in B cells (*Hem1$^{fl/fl}$Mb1CreMD4*) that are otherwise similar to Hem1-normal MD4+ controls (*Hem1$^{fl/fl}$MD4*) (Figure 2A).

Although all B cells shared a single antigen specificity, *Hem1$^{fl/fl}$Mb1CreMD4* again expressed higher baseline CD69 and higher CD69 after 24 hours α-IgM or α-IgD stimulation (Figure 2B, 2C). By 24 hours post stimulation, CD69 remained higher in *Hem1$^{fl/fl}$Mb1CreMD4* mice relative to *Hem1$^{fl/fl}$MD4* control mice following α-IgM but not α-IgD stimulation. Interestingly, expression of the inhibitor receptor CD23 was higher in *Hem1$^{fl/fl}$Mb1CreMD4* mice relative to *Hem1$^{fl/fl}$MD4* control mice (Figure 2D), possibly reflecting a compensatory response to strengthened BCR signaling following loss of Hem1. These results collectively suggest that increased basal and post-stimulatory BCR signaling following disruption of Hem1 is likely independent of BCR specificity (Model 2, Figure 8).

Hem1 deficient B cells exhibit increased apoptosis and decreased cell survival

We next evaluated whether disruption of Hem1 had any effects on resting or BCR activation induced apoptosis and survival. We cultured B cells from *Hem1$^{fl/fl}$Mb1Cre* mice and Hem1$^{fl/fl}$ controls for 16 hours in conditions of α-IgM stimulation, α-IgD stimulation, and no stimulation. We then measured apoptosis and cell death by flow cytometry using Annexin V, a

Caspase-3 stain, along with propidium iodide (PI), an intracellular nucleic acid dye, which only penetrates and stains dead cells with compromised cell membranes. Under all conditions, Hem1 KO samples had fewer $B220^{+}$Annexin $V^{-}PI^{-}$ viable B cells (Figure 3A, B). This correlated with increased $B220^{+}$Annexin $V^{+}PI^{-}$ apoptotic cells at baseline and following anti-IgD stimulation. Cell death was increased significantly in α-IgM stimulated samples (Figure 3C). In conditions of the weaker α-IgD antibody stimulation, or no stimulation, a larger proportion of Hem1 cells entered apoptosis.

Increased mitochondrial mass and reactive oxygen species in Hem1-deficient B cells

The activation state of B cells can profoundly influence their metabolism. Both signaling and proliferation patterns of activated B cells cause increased metabolic demands, which in turn cause changes in mitochondrial fission and ROS production (14-16). We evaluated mitochondrial function in *Hem1$^{fl/fl}$Mb1Cre* and *Hem1$^{fl/fl}$Mb1CreMD4* mice, along with *Hem1$^{fl/fl}$* and *Hem1$^{fl/fl}$MD4* controls, by two metrics: mitochondrial mass, which represents the total mass of mitochondria within a cell, and mitochondrial reactive oxygen species (ROS), which simultaneously enhance BCR signal strength and threaten B cell survival as toxic byproducts of mitochondrial metabolism (16). Cells were cultured for 24 hours with α-IgM antibody, LPS, or no stimulation, then subsequently dyed with MitoTracker Green (to measure mitochondrial mass) or MitoSOX Red (to measure mitochondrial ROS).

In *Hem1$^{fl/fl}$Mb1Cre* mice with randomly generated BCRs, mitochondrial mass was increased in all conditions (baseline, α-IgM, LPS) relative to *Hem1$^{fl/fl}$* controls (Figure 4A). Mitochondrial ROS was found to be increased in unstimulated and LPS-stimulated B cells, but not in B cells stimulated with α-IgM (Figure 4B). Contrastingly, in *Hem1$^{fl/fl}$Mb1CreMD4* mice with a monoclonal BCR repertoire, mitochondrial mass was found to be near-identical to *Hem1$^{fl/fl}$MD4* controls following all stim conditions (Figure 4C). Despite this, mitochondrial ROS remained elevated in unstimulated and LPS-stimulated B cells, and additionally in B cells stimulated with α-IgM (Figure 4D). Taken together, these results suggest that BCR engagement of Hem1-deficient B cells is equally able to induce mitochondrial fission when compared to Hem1-sufficient controls, but these cells are then unable to mitigate ROS accumulation. In the case of *Hem1$^{fl/fl}$Mb1CreMD4,* the amplifying effects of ROS may result in a stronger overall activation signal than *Hem1$^{fl/fl}$MD4*

controls, despite equal antigen affinity to an identical BCR. At the same time, ROS toxicity predisposes these cells to apoptosis, as we observed previously.

RNAseq reveals increased expression of genes associated with cell cycle, metabolism, and chromatin remodeling

We next evaluated how B cell-specific loss of Hem1 affected gene expression in purified B cells following BCR stimulation. RNA from FACS purified $B220^{+}CD93^{-}CD21^{hi}CD23^{lo}$ FO B cells was sequenced using Illumina chips and evaluated through KEGG analyses for differentially expressed genes. Principal component analyses (PCA) revealed that each genotype separated distinctly into two groups (Figure 5A). Expression of 746 genes were significantly increased and 515 genes were significantly decreased based on P-adjusted values in Hem1 deficient B cells, which grouped tightly according to genotype (Figure 5B). As expected, expression of *Nckap1l* and *Mb1* were both significantly decreased in Hem1-deficient B cells, confirming the genotypes were correct. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses revealed that disruption of Hem1 resulted in enrichment of genes associated with the cell cycle and cell division, including E2F and Myc target genes, genes involved in the G2-M checkpoint (Figure 5D and E). Loss of Hem1 in B cells was also noted to enrich genes involved in cell metabolism (PI3K, mTOR, oxidative phosphorylation, glycolysis, fatty acid metabolism), interferon signaling, DNA repair/UV response, and apoptosis (Figure 5E).

To validate these results, we repeated stimulation of magnetic bead purified $Hem1^{fl/fl}Mb1Cre$ B cells and $Hem1^{fl/fl}$ controls, using either α-IgM and α-CD40 to mimic a T-cell dependent immune response, or no stimulation. Next, we lysed these cells for RNA, then synthesized cDNA to perform RT-qPCR on selected genes of interest. In Hem1-disrupted B cells, we observed a marked upregulation of DNA repair genes and E2F transcription factor targets (Figure 7B), which may suggest poorly controlled proliferation and unchecked growth at multiple cell cycle stages (17). In addition, there were observed increases in genes associated with mTORC1 metabolism (Figure 7C), interferon response (Figure 7D), providing further evidence to a strongly activated, inflammatory phenotype for Hem1-disrupted B cells. We also noted increases in c-Myc targets (Figure 7E), important oncogenes associated with both rapid cellular proliferation and vulnerability to apoptosis under stress (18). Finally, we found increased expression of genes associated with cell growth and proliferation, particularly at the G2-M checkpoint (Figure 7F).

III. Discussion

Humans with loss-of-function variants in the *NCKAP1L* gene encoding HEM1 are severely immunodeficient yet prone to develop autoimmunity, with a disease phenotype characterized by poor antibody responses to immunization and increased autoantibody production. We previously found that mice with constitutive or B cell specific disruption of Hem1 exhibit B lymphopenia and poor T-independent antibody responses following immunization. However, B cell specific Hem1 disruption also resulted in increased autoantibody production, as well as increased B cell activation both basally and following BCR stimulation. In the current study, we addressed potential mechanisms as to why loss of Hem1 resulted in B lymphopenia and B cell hyperactivation. We found that Hem1-deficient B cells produced a highly sensitive, hyperactivated response to BCR activation, independent of BCR specificity or selection for BCR signal strength during development. This extreme antigen sensitivity appeared to profoundly reduce B cell survival, causing them to generate large amounts of ROS and exposing a high degree of vulnerability to apoptosis. Based on our results, we conclude that loss of Hem1 in B cells directly and profoundly altered these cells' response to antigen activation.

In healthy, naïve B cells, cytoskeletal actin plays a crucial role in maintaining spacing between copies of the BCR on the cell surface, preventing multiple receptors from drifting together and crosslinking before antigen binding (19). At the same time as it controls BCR interactions, cytoskeletal actin also serves as an important regulator of cellular proliferation, with its removal or inhibition leading to rapid, but dysfunctional cellular division (20, 21). In our Hem1-deficient model, in which actin formation is severely impacted at an early stage of the WRC, poor actin formation may cause failure of B cells to maintain distance between receptors. This may allow multiple BCRs on the same cell to crosslink inappropriately, causing these cells to produce strong activation signals despite weak or nonexistent antigen binding. The effects of unusually strong activation are carried out by upregulation of proliferative and growth-related genes as these cells mount unusual responses to weakly-binding antigens, potentially including host autoantigens.

It is likely that these cells' strong signals when faced with weak antigen binding are directly related to the depletion of mature B cells observed in Hem1-deficient mice. Following BCR activation, cells accumulate ROS, which further amplifies their unusually strong signal. At the same time, these cells rapidly upregulate genes related to metabolism and DNA repair, creating a

feedback loop which increases their sensitivity to apoptosis; and then further influenced by increased expression of c-Myc and its targets.

Overall, the effects of Hem1 disruption are fundamental and far-reaching, even when isolated to B cells alone. Future directions for this work may include quantification of signaling molecules within stimulated B cells via immunoblot or similar, imaging of the Hem1-deficient B cell synapse, and/or exploring the molecular mechanisms by which actin loss may cause increased signaling in B cells.

IV. Materials and Methods

Generation of *Hem1* cKO mice with fixed BCR

Hem1 floxed mice expressing B cell-specific Cre recombinase at the *Mb1* locus *($Hem1^{fl/fl}Mb1Cre^{+}$),* at generations 10-12 backcrossed on a C57BL/6J background, were bred to *Hem1* floxed mice expressing a transgenic BCR known as IgHEL *($Hem1^{fl/fl}MD4^{+}$)* (12). Mice were housed and maintained in a specific pathogen-free animal facility. Genotypes were screened and validated by PCR for each gene of interest. Male and female mice did not differ phenotypically, and were used equally in experiments. Experimental cohorts were selected from mice aged 8-24 weeks, using littermate controls expressing floxed *Hem1* but no Cre recombinase.

B Cell Purification

Because only B cells were affected by the conditional knockout of *Hem1*, experiments were most often run on total splenocytes. However, some applications, particularly RT-qPCR, required isolation of B cells alone. B cells were purified on a magnetic bead column using a pan-B cell isolation kit (Miltenyi Biotec cat. 130-104-443) according to kit manufacturer's instructions. Purity of B cells in resultant samples was estimated by flow cytometry (gated $CD19^{+}$) to be between 88-98%.

Cell Culture and B Cell Stimulation

Total splenocytes were harvested from *Hem1*$^{fl/fl}$ and *Hem1*$^{fl/fl}$*Mb1Cre*$^{+}$ mice. Cells designated "No Stim" controls were cultured in a 37°C incubator for 16-48 hours in complete B cell media, consisting of pre-mixed RPMI + GlutaMAX with 10% heat-inactivated ΔS FBS, HEPES, sodium pyruvate, pen strep; and with fresh 2-mercapethanol added immediately before use (13).

For stimulation, more samples were cultured for the same amount of time in the same media, with additions of stimulating functional antibody or protein as appropriate for the experiment. IgM stimulation was performed in unpurified cells using F(ab')2-goat anti-mouse IgM (Invitrogen cat. 31178) at strengths of 10 μg/mL (activation status, apoptosis analysis) or 5 μg/mL (RT-qPCR, proliferation, mitochondrial activity). In the case of purified B cells used for RT-qPCR, CD40L (Invitrogen cat. 14-0402-B6) was also added to the IgM stim media at equal concentration (5 μg/mL). IgD stimulation was performed with goat anti-mouse IgD (MD Bioproducts cat. 2057001) at 10 μg/mL. LPS stimulation was conducted with purified bacterial LPS at 5 μg/mL.

Apoptosis Analysis

Hem1$^{fl/fl}$ and *Hem1*$^{fl/fl}$*Mb1Cre* splenocytes were divided into three groups to be stimulated with α-IgM, α-IgD, or no stim, at concentrations listed above, then incubated at 37°C for 16 hours. At the end of incubation, cells were stained using B220 PB (Tonbo Biosciences cat. 75-0452), Annexin V-FITC and propidium iodide (Invitrogen cat. V13241) at 1:300, 1:200, and 1:500 dilutions, respectively. Data was acquired by flow cytometry using BD FACSCanto II and FACSDiva software, then analyzed in FlowJo 10.

RNAseq

B220^{+}CD93^{-}CD21^{hi}CD23^{lo} FO B cells were FACS purified from *Hem1*$^{fl/fl}$*Mb1Cre*$^{+}$ B cells and *Hem1*$^{fl/fl}$ control mice (N=4/group) and stimulated with 10 mg/ml α-IgM and Ifnγ for 12 hours in vitro in B cell growth media (see above). Cells were then lysed, RNA purified, and hybridized to Illumina chips (Illumina cat. FC-131-1024, FC-131-1096) using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara cat. 635025) protocol according to manufacturer instructions.

Mitochondrial Dyes

Samples were cultured in duplicate at 37°C for 24 hours, in stim conditions of 10 μg/mL α-IgM, LPS, or no stim. Immediately following incubation, all cells were stained with B220 PB (Tonbo Biosciences cat. 75-0452), then subsequently with either MitoTracker Green (Invitrogen cat. M7514) or MitoSOX Red (Invitrogen cat. M36008). Flow cytometry data was acquired as above, within one hour of staining.

Statistics

Single metric data was analyzed using the Student's 2-tailed unpaired *t* test with equal variance in GraphPad Prism 10. Multiple comparisons were analyzed using the same software for 2-way ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant in both analyses.

Study Approval

All studies involving animals were approved by the University of Washington's Institutional Animal Care and Use Committee.

V. Figures and Legends

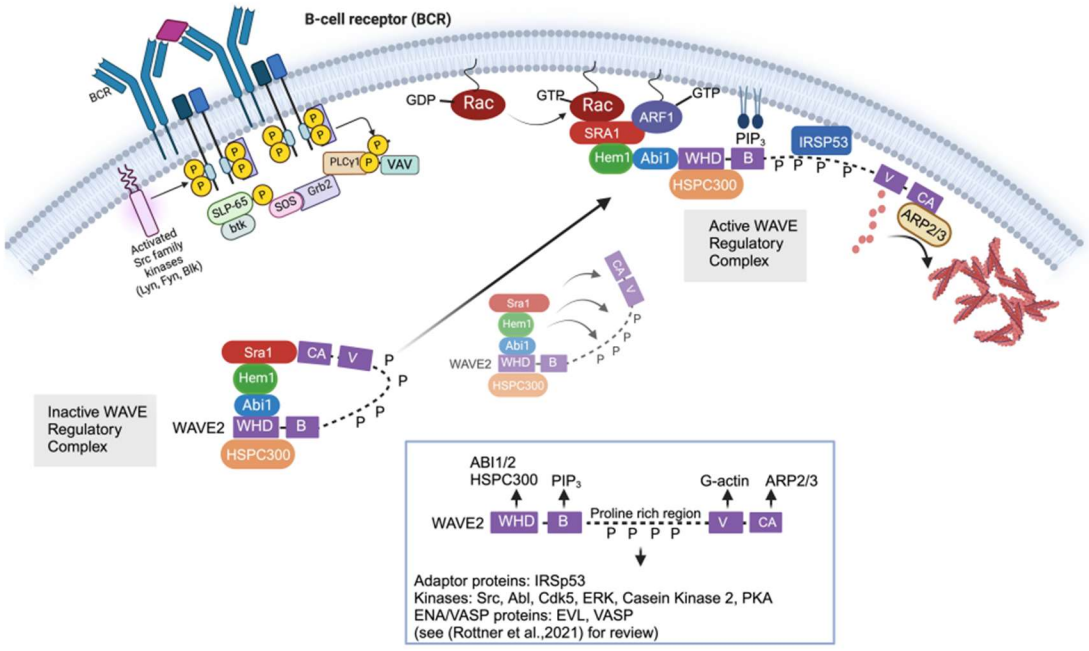

Figure 1. HEM1 plays a vital role in activation of the WRC and polymerization of F-actin.

Model depicting molecular activation of the WAVE regulatory complex. The c-terminal V, C, and A domains (collectively known as the VCA domain) interact with the "meander," maintaining the resting WAVE2 protein complex in a "closed" autoinhibitory state. Following receptor activation, the WRC migrates from the cytosol to the plasma membrane, where it interacts with PIP3. This causes allosteric release of the VCA domain from Sra1, allowing the VCA domain to interact with actin monomers and the ARP2/3 complex as is necessary for actin formation. *(From Christodoulou et al., Frontiers in Immunology 2024)*

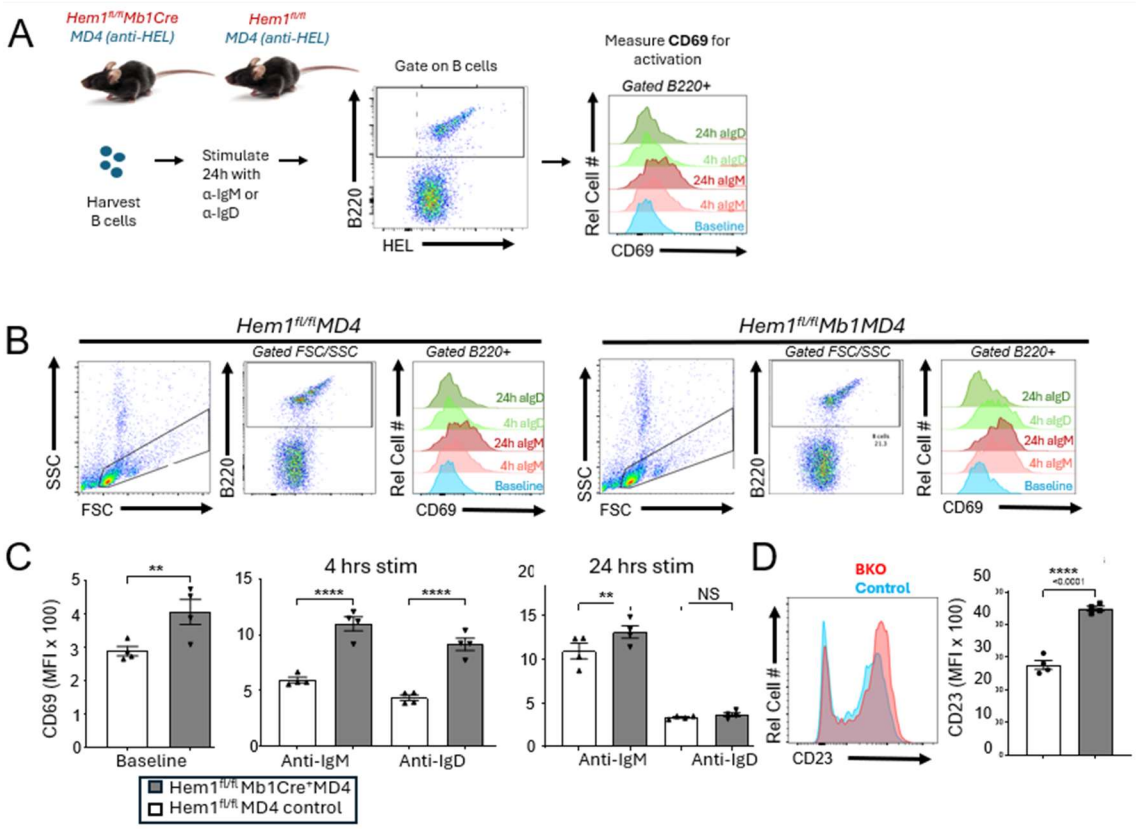

Figure 2. B cell specific disruption of Hem1 results in B cell hyperactivation independent of BCR specificity.

(A) Splenocytes were isolated from mixed-sex 12-24 week old *Hem1$^{fl/fl}$Mb1CreMD4* mice (n=3) and littermate *Hem1$^{fl/fl}$MD4* controls (n=3), then cultured for up to 24 hours in 10 µg/ml anti-IgM, anti-IgD, or no stim conditions. Splenocytes were stained with fluorescent-conjugated antibodies against B220, HEL, the activation marker CD69, and the inhibitory marker CD23, then acquired by flow cytometry. CD69^{+} expression was measured on gated B220^{+} cells at baseline, 4 hours, and 24 hours post-stimulation. (B) Representative flow cytometric dot plots of MD4^{+} WT (*top left*) and Hem1-disrupted MD4^{+} (*top right*) specimens. (C) Bar graphs depicting increased median fluorescence of CD69 in Hem1-deficient B cells, indicating higher activation status. (D) Representative flow cytometric overlay and bar graph depicting increased fluorescence of regulatory CD23 in Hem1-deficient B cells as a possible compensatory mechanism. Data is representative of 2 experiments ($n \geq 15$ per group). Each data point corresponds to an individual mouse. Data represent mean ± SEM and were analyzed via unpaired Student's t test. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001.

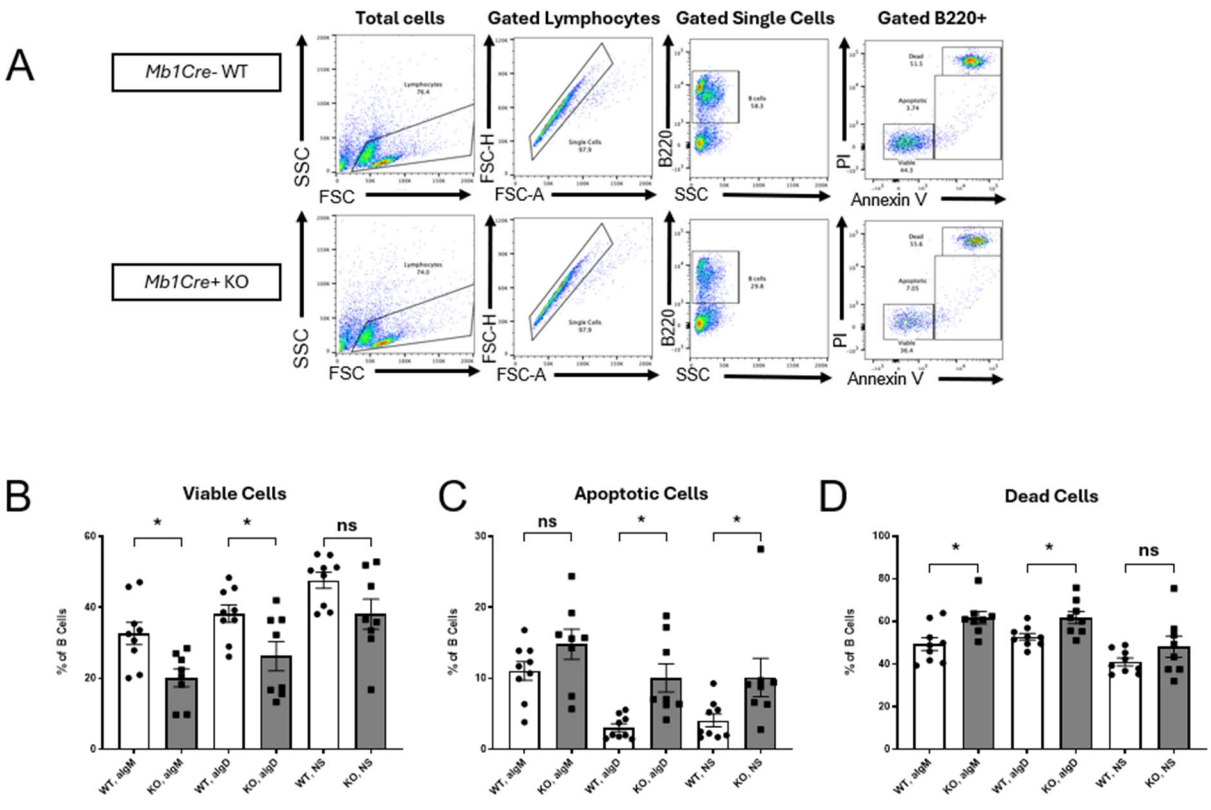

Figure 3. Hem1 deficiency in B cells results in increased apoptosis and cell death.

Total splenocytes were isolated from mixed-sex 12-24 week old *Hem1*$^{fl/fl}$*Mb1Cre* and *Hem1*$^{fl/fl}$ littermate controls (n= 8/group), then cultured 16 hours with α-IgM, α-IgD, or no stim. B220^{+} B cells were evaluated for apoptosis and death by staining with Annexin V and PI, followed by flow cytometry. (A) Shown are representative dot plot histograms of *Hem1*$^{fl/fl}$ (*top*) and *Hem1*$^{fl/fl}$*Mb1Cre* (*bottom*) specimens, with gating strategy. (B) Bar graphs showing the proportion of B220^{+}Annexin V^{-}PI^{-} viable cells at 16 hours post-stimulation. (C) Bar graphs showing the proportion of B220^{+}Annexin V^{+}PI^{-} apoptotic cells at 16 hours post-stim. (D) Bar graphs showing the proportion of B220^{+}Annexin V^{+}PI^{+} dead cells at 16 hours post-stimulation. Data are pooled from 2 independent experiments (n ≥ 15 per group). Each data point corresponds to an individual mouse. Data represent mean ± SEM and were analyzed via one-way ANOVA with Tukey's multiple comparison test. *P < 0.05.

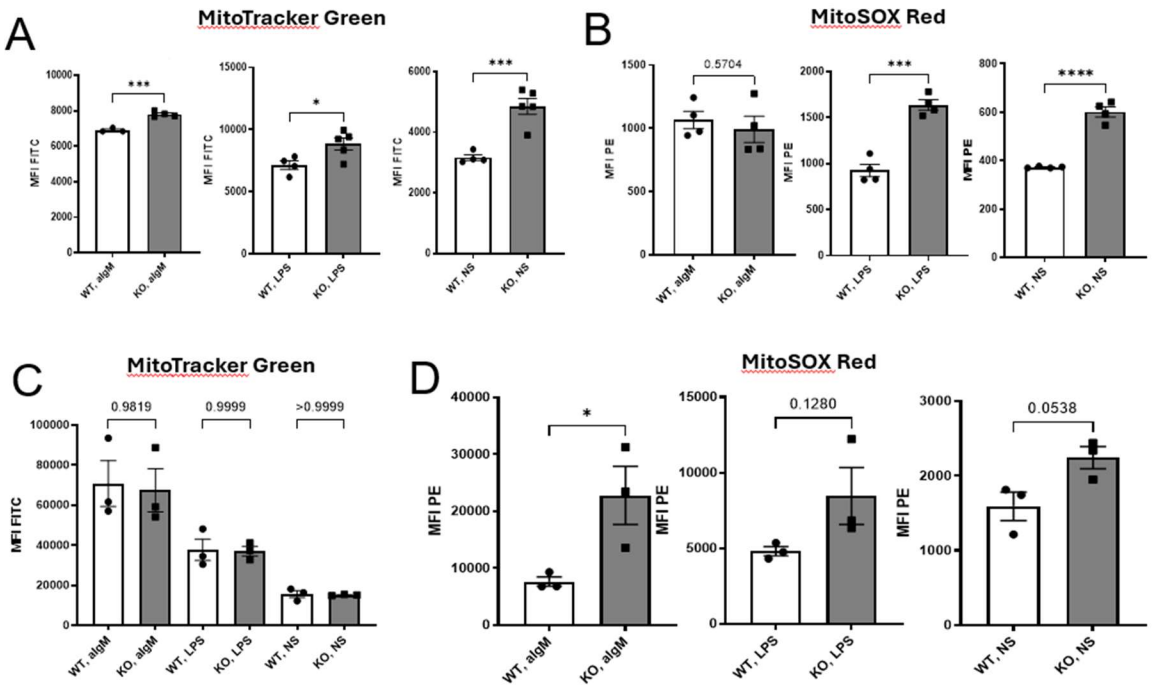

Figure 4. Increased mitochondrial mass and ROS in B cells following disruption of Hem1.

Total splenocytes were harvested from *Hem1*$^{fl/fl}$ *Mb1Cre* and *Hem1*$^{fl/fl}$ mixed-sex mice of 8-16 weeks of age. Cells were stimulated with α-IgM, LPS, or no stim, then stained with fluorescent conjugated antibodies against B220 and either MitoTracker Green or MitoSOX Red to evaluate mitochondrial activity. (A) Shown are bar graphs depicting median fluorescence levels (MFI) of MitoTracker Green on gated B220^{+} cells with and without stimulation. (B) Shown are bar graphs depicting MFI of MitoSOX Red with and without stimulation. (C, D) Splenocytes were isolated from mixed-sex 12-24 week old *Hem1*$^{fl/fl}$*Mb1CreMD4* mice (n=3) and littermate *Hem1*$^{fl/fl}$*MD4* controls (n=3), then cultured for 12 hours in 10 μg/ml anti-IgM or LPS or no stim conditions. Cells were then stained with fluorescent conjugated antibodies against B220 and either MitoTracker Green or MitoSOX Red to evaluate mitochondrial activity. (C) Shown are bar graphs depicting MFI of MitoTracker Green on gated B220^{+} cells with and without stimulation. (D) Shown are bar graphs depicting MFI of MitoSOX Red with and without stimulation. Data is representative of 2 experiments ($n \geq 8$ per group). Each data point corresponds to an individual mouse. Data represent mean ± SEM and were analyzed via unpaired Student's t test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.0001$.

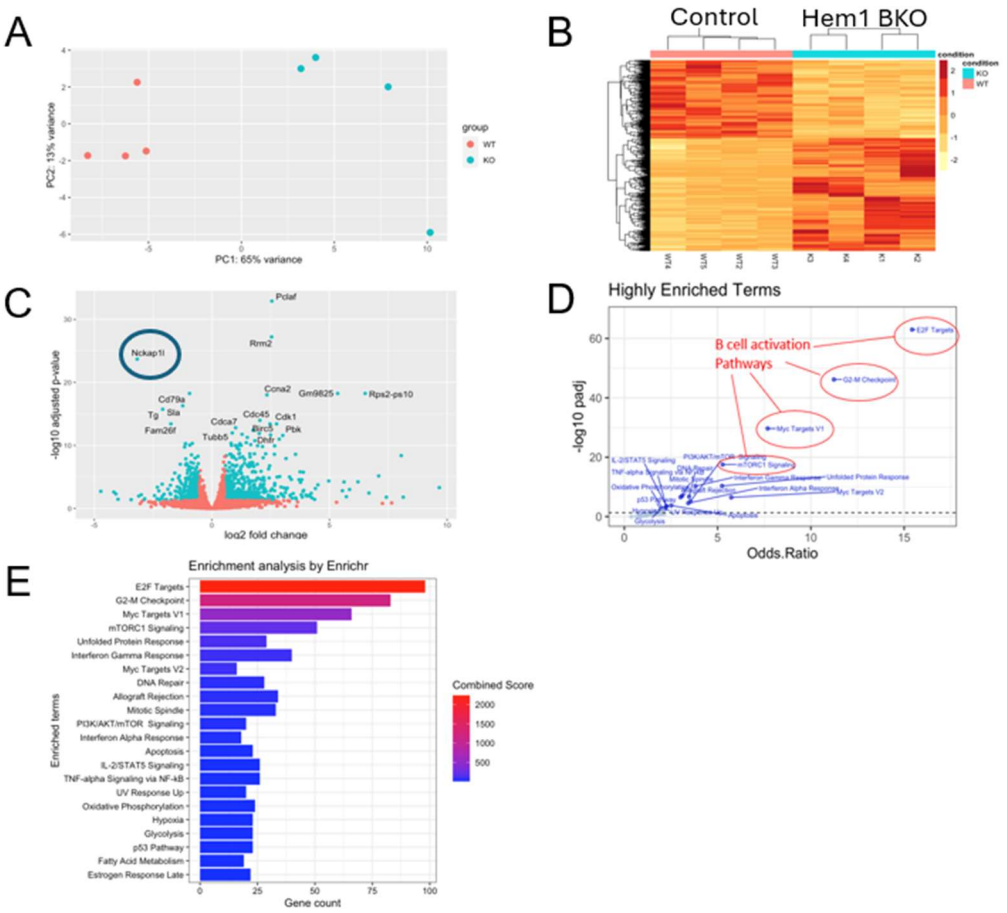

Figure 5. RNAseq reveals increased expression of genes associated with cell cycle, metabolism, and chromatin remodeling in B cells following disruption of Hem1.

Total splenocytes from *Hem1*$^{fl/fl}$*Mb1Cre* and *Hem1*$^{fl/fl}$ mice (n=4/group) were FACs sorted based on B220^{+}CD93^{-}CD21^{hi}CD23^{lo} FO B cells, followed by stimulation with α-IgM and IFNγ (N=4/group). RNA was isolated and processed for RNAseq by staff at Benaroya Research Institute. RNA was hybridized to Illumina arrays. (A) Principal components analysis showing grouping of mice by genotype. (B) Heat map of upregulated/downregulated genes, once again demonstrating strong grouping by genotype. (C) Volcano plot depicting fold change (x-axis) and significance (y-axis) of differentially expressed genes. *Nckap1l* and *Cd79a* are both downregulated in *Hem1*$^{fl/fl}$*Mb1Cre* mice with a high degree of significance, validating the RNAseq study. (D) Strong representation of B cell growth and activation pathways among the most significantly upregulated genes with the greatest effect sizes. (E) E2F targets, Myc targets, G2-M checkpoint genes, metabolism, DNA repair, and interferon response genes are among the most commonly enriched genes in *Hem1fl/flMb1Cre* samples.

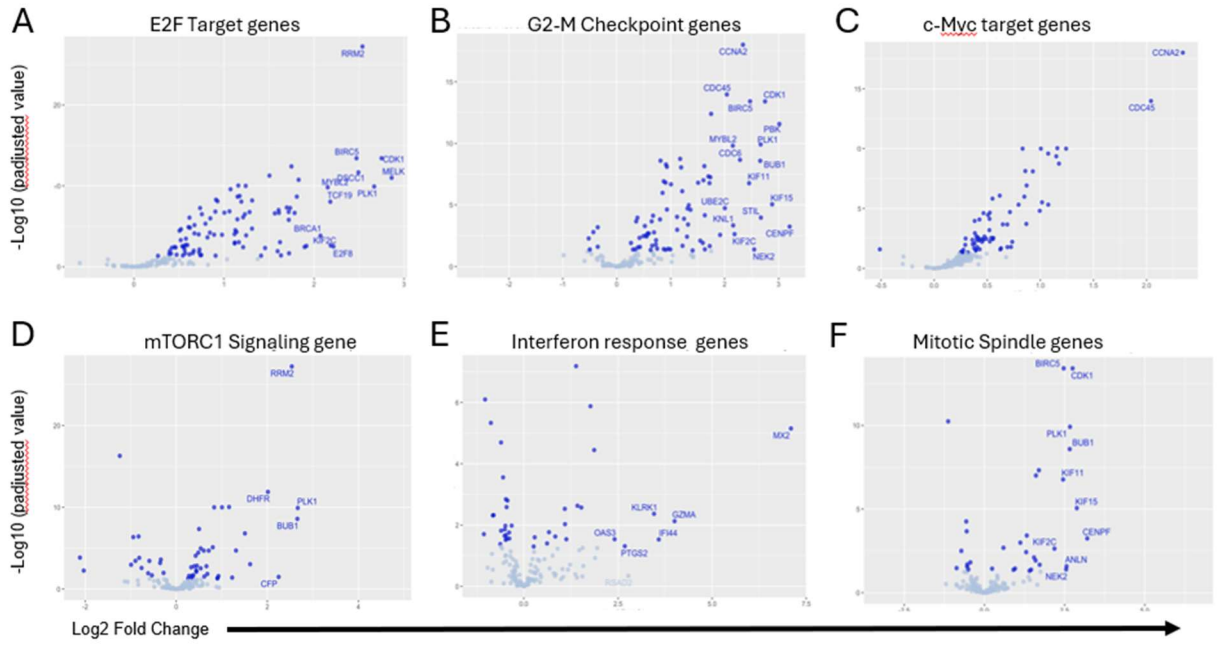

Figure 6. PCA analyses reveals enrichment for specific genes associated with pathways including E2F, mTORC1, Myc, mitotic spindle, interferon response, and G2-M checkpoints.

Following RNASeq, we performed database KEGG analyses to sort genes into functional groups. (A-F) Volcano plots depicting fold change (x-axis) and significance (y-axis) of enriched terms in Hem1 deficient B cells vs control B cells. (A) Select E2F genes upregulated in Hem1 deficient B cells. (B) Select upregulated genes associated with the G2-M checkpoint. (C) Select upregulated c-Myc target genes suggest a proliferative response to B cell stimulation. (D) Select increased mTORC1 signaling genes associated with hypermetabolic phenotype. (E) Select increased interferon response genes reflect a strong inflammatory response. (F) Select upregulated genes associated with the mitotic spindle suggest increased rates of mitosis.

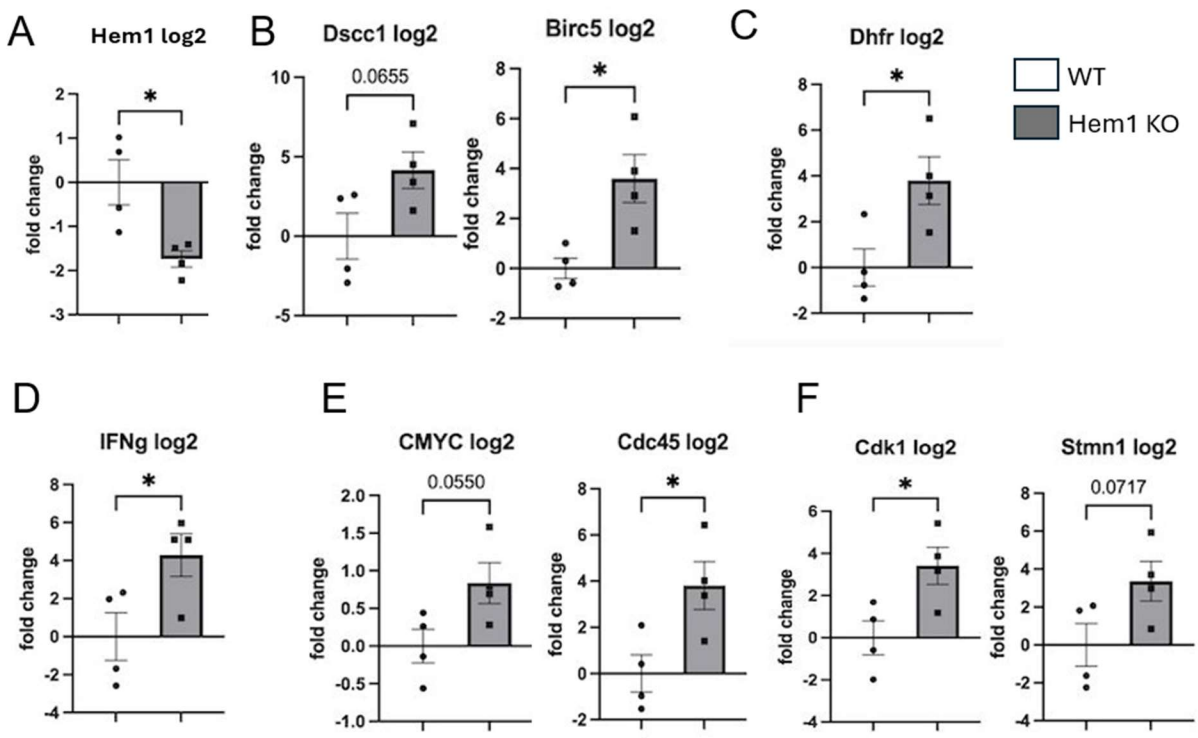

Figure 7. Realtime PCR confirms alterations in specific genes associated with interferon response, cell cycle, and metabolism.

Magnetic bead purified B cells from 12 week old *Hem1*$^{fl/fl}$*Mb1Cre* and *Hem1*$^{fl/fl}$ mice (N=4/group) were stimulated with 5 μg/mL α-IgM and α-CD40 for 16 hours, then RNA purified and cDNA synthesized for realtime quantitative PCR application. (A-F) Bar graphs depicting fold change in gene expression of selected genes previously identified as enriched in *Hem1*$^{fl/fl}$*Mb1Cre,* as calculated by ΔΔCt (22). (A) Downregulation of Hem1 validates mouse genotypes. (B) E2F target genes associated with DNA replication and chromatin binding are upregulated in *Hem1*$^{fl/fl}$*Mb1Cre.* (C) Dihydrofolate reductase, a metabolic protein and mTORC1 target, is transcriptionally upregulated in *Hem1*$^{fl/fl}$*Mb1Cre.* (D) Interferon gamma upregulation suggests a stronger inflammatory response to stimulation. (E) c-Myc and its targets are enriched in *Hem1*$^{fl/fl}$*Mb1Cre,* suggesting an increased proliferative response to stimulation. (F) Genes associated with mitosis, particularly surrounding the transition between cell cycle phases G2/M, are enriched in *Hem1*$^{fl/fl}$*Mb1Cre.* Data represent mean ± SEM and were analyzed via unpaired Student's t test. *P < 0.05.

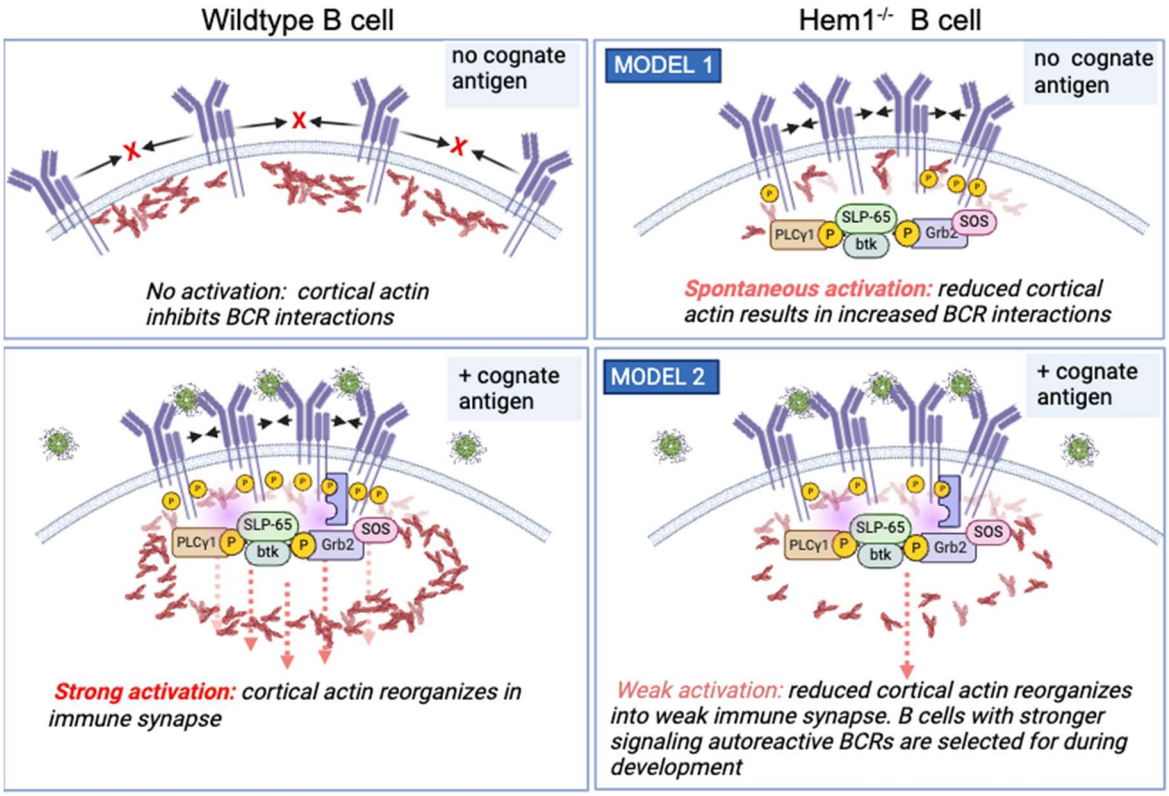

Figure 8. Model of B cell Activation in Hem1 Deficient B cells.

(*top left*) In naïve B cells before encountering antigen, crosslinking of resting BCR complexes is prevented by cortical actin spacing. (*bottom left*) Antigen binding causes redistribution of cortical actin as BCRs group into microclusters to form the immune synapse, launching a cascade of signaling molecules resulting in B cell activation. (*top right*) In our first proposed model, loss of cortical actin following disruption of Hem1 allows for increased spontaneous BCR diffusion and interactions, resulting in increased B cell activation even before antigen stimulation. (*bottom right*) In a second, alternative model, disruption of Hem1 causes weak immune synapse formation, encouraging selection for traditionally stronger-signaling autoreactive BCRs during B cell development. *(From Christodoulou et al., Frontiers in Immunology 2024)*

VI. References

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